出芽酵母を用いた遺伝性大腸癌の新しい遺伝子診断法の開発

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研究目的

本研究は、遺伝性大腸癌のうち遺伝性非ポリープ性大腸癌(HNPCC)及び家族性大腸腺腫症家系スクリーニングのための簡便な方法を開発する事を目的とする。このため、（1）HNPCCの原因遺伝子であるミスマッチ修復遺伝子群を出芽酵母に発現し、酵母自身のミスマッチ修復系におよぼす影響を指標としたアッセイ系を開発する。（2）APC遺伝子の変異は、そのアミノ酸コード領域にフレームシフトまたはストップコドンにを生じる変異であることから、APC cDNA断片を下流のマーカー遺伝子に結合し、酵母に発現させたときにマーカー遺伝子が発現するか否かを指標とするアッセイ系を開発する。（3）臨床検体（該当患者由来の末梢血）について、（1）および（2）のアッセイ系を使用、その有用性について検討する。

研究実施計画

(1)　出芽酵母を用いたDNAミスマッチ修復遺伝子およびAPC遺伝子の診断系開発
発現ベクターを用いて、ミスマッチ修復遺伝子群のアッセイ系を開発する。また、APC遺伝子のフレームシフトまたはストップコドン変異を検出できるアッセイ系を開発する。
(2)　酵母のアッセイ系を用いた臨床検体のスクリーニング
収集した臨床検体を用いて、(1)のアッセイ系をテストする。

研究成果概要

(1)　DNAミスマッチ修復遺伝子およびAPC遺伝子の診断システムの開発

(1-a)ヒトDNAミスマッチ修復遺伝子の診断システムの開発
*ADH*プロモーターによって発現する各種*hMSH2, hMLH1, hPMS2*発現ベクターを作成した。このうち*hMLH1*を野生型出芽酵母に発現した場合、酵母のミスマッチ修復系が競合阻害されることが判明、診断用アッセイの指標になる可能性が示された。酵母のミスマッチ修復系の異常を検出するための新しいマーカーとして、GT繰り返し配列をLacZ遺伝子の翻訳開始部位の下流に挿入したリポーターベクターを開発した。hMLH1発現ベクターを改良し、*hMLH1* PCR断片を容易に酵母に発現できるgap repair systemを開発した。このgap repair systemと新しいリポーターベクターを用いて、遺伝性非ポリープ性大腸がん(HNPCC)家系から見つかった既知のhMLH1変異(25種類)について、酵母ミスマッチ修復系の競合阻害性について検討、23種類の変異のうちミスセンス変異を含む8種類の変異はこの競合阻害性を示さず、正常*hMLH1*と区別できた。研究成果は国際学術論文として公表予定である。

(1-b)*PGK*プロモーターによってAPC遺伝子断片を発現するプラスミドを複数作成した。各種APC 断片の下流に各種マーカー遺伝子を挿入し、APC 断片とマーカー遺伝子から翻訳される融合タンパク質を酵母に発現させた。その結果、*URA3*遺伝子と融合した場合に、URA3機能が保持されることが判明し、アッセイ用ベクターとした。(1)同様に*APC* PCR断片を容易に酵母に発現できるgap repair systemを開発し、既知の*APC*変異(そのほとんど全てが、ナンセンス変異やフレームシフト変異である)についてアッセイした。その結果、全ての変異*APC*断片を挿入した場合、*URA3*融合タンパク質が発現せず(Ura-)、正常APC断片(Ura+)と区別できた。その詳細は国際学術論文として公表している。

(2) 臨床検体の収集および目的遺伝子断片の増幅

遺伝性大腸癌の患者検体(末梢血液)を収集し、核酸(RNA, DNA)を抽出、PCRにて目的遺伝子を増幅し、上記(1-a), (1-b)のアッセイ系を用いて*hMLH1*および*APC*遺伝子変異について検索した。その結果、これまでに1家系の*hMLH1*のミスセンス変異および8家系のAPC変異を同定している。その詳細は国際学術論文として公表している。また、*APC*変異の検出ついては(2)のアッセイは発症前診断にも有用であった。現在さらに家系を収集してアッセイ系の有用性について検討している。

Novel method for detection of heterozygous truncating mutations in the familial adenomatous polyposis and familial breast and ovarian cancer genes, *APC* and *BRCA1*, using a rapid screening assay in *Saccharomyces cerevisiae*.

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The identification of novel germ-line mutations in tumor suppressor genes presents a major difficulty in their initial characterization, as well as in the adaptation of reliable and effective approaches to clinical diagnostics. The large size of many of these genes and the fact that germ-line mutations are heterozygous have complicated analysis based exclusively on nucleotide sequencing. We have previously described a functional assay to detect germ-line mutations in *p53*, a gene that is affected primarily by missense mutations, and whose function as a transcriptional activator can be tested in yeast[1]. While the functional properties of other cancer susceptibility genes are not well understood, many of these are disrupted primarily by truncating mutations. Virtually all mutations in the colon cancer gene *APC* [2-5] and 80% of mutations in the breast cancer predisposition gene *BRCA1* [6] consist of nonsense or frameshift mutations, leading to the development of screening assays based on the *in vitro* production of truncated peptides, so-called PTT assays[7-11]. These methods are reliable and effective, but require significant levels of technical expertise and interpretation of results. Here we describe a simple yeast-based method, which makes use of homologous recombination in *Saccharomyces cerevisiae* to separate alleles and involves the production of a marker fusion protein to test for truncating mutations.

The stop codon scan (SC assay) benefits from two advantages of yeast systems. First, the ability to synthesize fusion proteins with orotidine-5'-phosphate (OMP) decarboxylase encoded by the *URA3* gene and second, the use of gap repair and homologous recombination to efficiently insert a PCR-generated sequence into this fusion construct and separate the products of different alleles. The outline of the assay is summarized in Fig. 1. We constructed a centromeric yeast expression vector, pCI-HA(URA3)-2, with two selectable markers: *LEU2* and *URA3* (codons 5-267), which complement the genetic defects of the YPH499 strain, allowing growth in the absence of

leucine and uracil. The *URA3* gene is driven by the strong *PGK* promoter, tagged at the N-terminus by hemagglutinin (HA), and interrupted by a *Bam*HI site to allow introduction of exogenous DNA fragments. To test whether fusion of proteins to the N-terminus of *URA3* preserves the Ura^+ phenotype, 15 different coding sequences of 0.8-3.4kb in size, derived from 7 different genes were inserted in-frame. All insertions demonstrated the Ura^+ phenotype, confirming that this selectable marker is not disrupted by N-terminal fusion to a variety of protein domains (data not shown). The Ura^+ phenotype was dependent on use of the correct upstream promoter and translational initiation codon, as demonstrated by its loss following placement of the *URA3* fragment in the reverse orientation, by insertion of 4bp into the *Bam*HI site, or by insertion of exogenous DNA fragments containing an out-of frame insertion or in the reverse orientation. Synthesis of the expected full length *URA3* fusion protein from Ura^+ colonies was confirmed by immunoblotting analysis using anti-HA antibody. As predicted, insertion of out-of-frame sequences into pCI-HA(URA3)-2 led to the expression of HA-positive truncated fusion proteins in Ura^- colonies (data not shown).

To test the SC assay in detecting truncating mutations in *BRCA1*, the coding sequence was divided into three overlapping fragments, which were amplified by RT-PCR and inserted in-frame into the *Bam*HI site of the pCI-HA(URA3)-2 vector. The resulting constructs, pCI-BR1a, b and c, showed preservation of the Ura^+ phenotype following introduction into yeast. The plasmids were converted into the corresponding "gap vectors", pCI-BR1ag, pCI-BR1bg and pCI-BR1cg, by removing most of the *BRCA1* insert, leaving 100 bp of flanking *BRCA1* sequence to allow for homologous recombination (see Fig. 1b and Methods). The three corresponding *BRCA1* fragments were then amplified by RT-PCR from peripheral blood mononuclear cells of patients with known *BRCA1* truncating mutations and controls. Cotransformation of unpurified PCR products and corresponding linearized gap vectors into yeast allowed

homologous recombination and growth of leucine auxotrophs containing the recircularized plasmid. Twenty five independent transformants were assayed for growth in the absence of uracil. Following gap repair with PCR products derived from control lymphocytes, 88-100% of transformants were Ura^+, demonstrating efficient homologous recombination of the *BRCA1* fragment and reconstitution of the *URA3* fusion protein (Table 1). The small fraction of Ura^- transformants is presumably due to infidelity of the Taq DNA polymerase and to recombination error, as described previously[1]. In contrast, PCR products derived from the lymphocytes of patients with known heterozygous *BRCA1* mutations[11] led to 44-64% Ura^+ transformants. These included specimens from two patients (#231 and #253) with a heterozygous 2bp deletion at codon 23 (the so-called 185delAG mutation) (gap vector pCI-BR1ag), one patient (#99) with a 2bp deletion at codon 327 (gap vector pCI-BR1bg), one patient (#364) with a nonsense mutation at codon 563 (gap vector pCI-BR1bg), and one patient (#250) with a 1 bp insertion at codon 1756 (the so-called 5382insC mutation) (gap vector pCI-BR1cg). Another 6 samples that only contained *BRCA1* polymorphisms[11] scored as wild-type (Table 1). The distribution of Ura^+ and Ura^- colonies derived from specimens with or without *BRCA1* truncating mutations was reproducible and non-overlapping (Fig. 2), demonstrating the reliability of this assay for diagnostic purposes.

To test the ability of the SC assay to detect unknown mutations, we chose the familial polyposis gene *APC*, which is inactivated exclusively (~93%) by truncating mutations located within the N-terminal 60% of the coding sequence[12]. This portion of the *APC* cDNA was divided into two fragments, APCa and APCb, which contains the so-called mutation cluster region (MCR)[13], was further divided into two overlapping fragments, APCc and APCd. As expected, in-frame insertion of these fragments into the *Bam*HI site of pCI-HA(URA3)-2 preserved the Ura^+ phenotype. We then analyzed 24 individuals derived from six unrelated families with familial polyposis (FAP) (Table 2).

Analysis of the MCR region for 6 patients (individuals I-1 from Families B, D, E and F; individual I-7 from Family C; individual II-2 from Family A) who were clinically diagnosed as affected, using gap vector pCI-APCbg, yielded yeast transformants, half of which retained the Ura^+ phenotype (mean 49%, range 38-60%), consistent with the presence of a heterozygous truncating mutation (Table 2). To identify the precise location of each mutation, gap repair assays were performed using the internal gap vectors, pCI-APCcg and pCI-APCd (*NdeI*/*NsiI* digest): individual II-2 from Family A and individual I-1 from Family B, scored positive for mutations within fragment APCc, but not APCd, whereas the converse was true for individual I-7 from Family C and individuals I-1 from Families D-F. This analysis was extended to the remaining 18 members of these families, identifying 5 individuals as having heterozygous truncating mutations within the same fragment as the proband (Table 2). Six independent Ura^- colonies were pooled and subjected to direct nucleotide sequencing. The separation of *APC* alleles resulting from the gap repair assay made it possible to specifically analyze the mutant allele, avoiding the difficulties inherent in sequencing heterozygous mutations. All specimens scored as positive by SC assay were found to have truncating *APC* mutations: a 4bp deletion at codon 929 (3765del4) in Family A, a 1bp insertion at codon 938 (2831insT) in Family B, a 2bp deletion at codon 1249 (3765del2) in Family C, a 5bp deletion at codon 1309 (3945del5) in both Families D and E, and a 1bp deletion at codon 1322 (3983delA) in Family F (Table 2). Analysis of other members from each family demonstrated a complete concordance between the results of the SC assay and direct sequencing analysis (Table 2).

The mutational analysis that we describe here is comparable in its efficacy to previously described PTT techniques that involve PCR-amplification of gene fragments, followed by *in vitro* transcription-translation and resolution of encoded peptides by SDS-PAGE. However, the yeast-based SC assay provides a number of important technical

advantages compared to these *in vitro*, gel-based assays. Among these are (i) the ability to analyze larger (~3.5kb) DNA fragments, which reduces the number of PCR reactions required to scan an entire gene for truncating mutations, (ii) the ability to detect mutations that arise adjacent to the PCR-primers, which minimizes the overlap required between fragments, (iii) the separation of alleles, which greatly simplifies confirmation of heterozygous mutations by nucleotide sequencing, (iv) avoidance of the requirement for radioisotopes and for protein gel-electrophoresis. From a technical standpoint, the SC assay requires few manipulations and the time required to perform the assay (four days) reflects that required to clearly visualize yeast colonies after sequential plating on leucine and uracil deficient plates. The use of a mutational screening test based on the detection of truncating mutations also has important advantages in the adaptation of such approaches to clinical diagnostics. Protein truncating mutations comprise the majority of inactivating mutations for a number of important cancer predisposition genes, including *BRCA1*, *BRCA2*, *APC*, mismatch repair genes, and potentially *ATM*. Furthermore, the difficulty in interpreting missense mutations precludes their use in most clinical diagnostics. The SC assay thus provides a rapid and reliable test that can be readily adapted to detect heterozygous truncating mutations in cancer predisposition genes and other genes implicated in human disease.

Methods

Plasmid construction. The plasmid pCI-HA(URA3)-2 was constructed as follows: a fragment spanning nucleotide-number (nt.) 423 to 1239 of the plasmid pRS316[14] (GenBank U03442), which contains *URA3* coding sequence from codon 5 to the natural termination codon, was amplified by PCR using a set of primers containing a *Bam*HI site or a *Bgl*II site at the 5' end. The *Bam*HI/*Bgl*II fragment was inserted in-frame into the *Bam*HI site of the plasmid pRS-PGK[15] to produce pCI-HA(URA3). This vector was digested with *Nsi*I and *Pst*I and religated to produce pCI-HA(URA3)-2 (Fig. 1a).

Three fragments spanning nt. 96 to 908 (BRCA1a), nt. 789 to 4214 (BRCA1b) and nt. 4089 to 5708 (BRCA1c) of the *BRCA1* cDNA (GenBank U14680), were amplified and inserted in-frame into the *Bam*HI site of the pCI-HA(URA3)-2 to produce pCI-BR1a, pCI-BR1b and pCI-BR1c, respectively (Fig. 1b). Four fragments spanning nt. 19 to 1977 (APCa), nt. 1978 to 5256 (APCb), nt. 1978 to 3570 (APCc) and nt. 3571 to 5256 (APCd) of the *APC* cDNA (GenBank M74088), were amplified and inserted in-frame into the *Bam*HI site of pCI-HA(URA3)-2 to produce pCI-APCa, pCI-APCb, pCI-APCc and pCI-APCd, respectively (Fig. 1b). All the vectors described above result in a Ura^+ phenotype, following introduction into YPH499 strain. The gap vectors, pCI-BR1ag, pCI-BR1bg, pCI-BR1cg, pCI-APCag, pCI-APCbg and pCI-APCcg (Fig. 1b) are identical to pCI-BR1a, pCI-BR1b, pCI-BR1c, pCI-APCa, pCI-APCb and pCI-APCc, respectively, except that the central portions of the inserted fragments, BRCA1a, BRCA1b, BRCA1c, APCa, APCb and APCc, between nt. 183 to 827, nt. 888 to 4111, nt. 4215 to 5609 (GenBank U14680), nt. 109 to 1899, nt. 2054 to 5201 and nt. 2086 to 3489 (GenBank M74088), were replaced by the unique restriction sites, *Bgl*II, *Stu*I/*Bam*HI/*Sma*I, *Bgl*II, *Bgl*II, *Nsi*I and *Bgl*II, respectively. All the gap vectors except pCI-APCbg were produced by PCR using ExTaq (TAKARA) and the original plasmids with full-length insertion as templates, followed by ligation using the unique restriction sites described above and transformation of *E. coli* (DH5α). The pCI-APCbg was obtained by removing the central portion of APCb fragment of the pCI-APCb using two *Nsi*I sites.

PCR. For *BRCA1* and *APC* analysis, genomic DNA and/or total cellular RNA was isolated from EBV-immortalized cell lines or peripheral blood mononuclear cells. cDNA was synthesized by using a First-Strand cDNA Synthesis Kit (Pharmacia). BRCA1a-c and APCa (see Fig. 1b) were amplified from cDNA. BRCAb and APCb-d were amplified from genomic DNA. Primers for amplification of *BRCA1* fragments were

5'-GAAAGTTCATTGGAACAGAAAGAA-3' and

5'-ACCCTGATACTTTTCTGGATG-3' for BRCA1a,

5'-CCCAGATCTGCTGCTTGTGAATTTTCTGAG-3' and

5'-CCCAGATCTTAAGTTTGAATCCATGCTTTG-3' for BRCA1b, and

5'-ATGAGGCATCAGTCTGAAAGC-3' and

5'-GTAGTGGCTGTGGGGGATCT-3' for BRCA1c.

Primers for amplification of *APC* fragments were

5'-ATGGCTGCAGCTTCATATGAT-3'

and 5'-CTGTGGTCCTCATTTGTAGC-3' for APC1a,

5'-CAAATCCTAAGAGAGAACAAC-3' and

5'-GTCCATTATCTTTTTCACACG-3' for APCb,

5'- CAAATCCTAAGAGAGAACAA-3' and

5'-GGCATATTTTAAACTATAATC-3' for APCc, and

5'-ACAGATATTCCTTCATCACAG-3' and

5'- GTCCATTATCTTTTTCACACG-3' for APCd. All PCR fragments were obtained by using ExTaq polymerase (TAKARA). Details of the PCR parameters are available from the authors upon request.

Yeast transformation. The yeast strain used in this study was YPH499 (*MATa, ura3-52, lys2-801^{amber}, ade2-101^{ochre}, trp1Δ63, his3Δ200, leu2Δ1*) (Stratagene). Competent yeast cells were prepared by lithium acetate (LiOAc) treatment of the strain cultured in YPD liquid medium[1] and were stored at -80°C in the presence of 5% DMSO until use. Frozen competent yeast retain high transformation efficiency for at least three months. Gap repair assays were performed by cotransformation of unpurified PCR product (~200ng) and linearized gap vector (~30ng) by the LiOAc method[16] with minor modifications[1]. To analyze the PCR fragments, BRCA1a, BRCA1b, BRCA1c, APCa, APCb, APCc, and APCd, linearized gap vectors, pCI-BR1ag (*Bgl*II digest), pCI-BR1bg

(*Bam*HI/*Sma*I digest), pCI-BR1cg (*Bgl*II digest), pCI-APCag (*Bgl*II digest), pCI-APCbg (*Nsi*I digest), pCI-APCcg (*Bgl*II digest), and pCI-APCg (*Nde*I/*Nsi*I digest) were used, respectively. Transformants were selected on synthetic complete medium (SC) lacking leucine (SC-Leu); 25 transformants were then assayed for the Ura^+ phenotype by growth on SC lacking leucine and uracil (SC-Leu-Ura). If more than 85% of transformants are Ura^+, the sample is scored as homozygous wild-type, whereas if all transformants are Ura^-, the sample is scored as homozygous mutant. If 40-50% of colonies are Ura^+, the samples is scored as heterozygous for a truncation mutant.

Sequencing. Yeast DNA extraction from pooled Ura^- transformants was described previously[17]. Template *APC* fragments were amplified as described above and were sequenced using CircumVent™ Thermal Cycle Dideoxy DNA Sequencing Kit (New England BioLabs). Appropriate *APC* specific oligonucleotides were used as sequencing primers after end-labeled by [γ-^{33}P]dATP.

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Figure legends

Fig. 1 Schematic representation of SC assay.

a. pCI-HA(URA3)-2 vector. In-frame insertion of a coding sequence of interest into the unique *BamHI* site results in constitutive expression of an HA-tagged *URA3* fusion protein, driven from the *PGK* (3-phosphoglycerate kinase) promoter. In addition, the vector contains the *PGK* terminator downstream of the *URA3* fragment, the *LEU2* gene as a second selectable marker, and *CEN* and *ARS* for stable low copy number replication. The derived gap vector lacks the central portion of the inserted fragment, which can be replaced by a PCR-generated fragment inserted by homologous recombination, using the remaining flanking sequences.

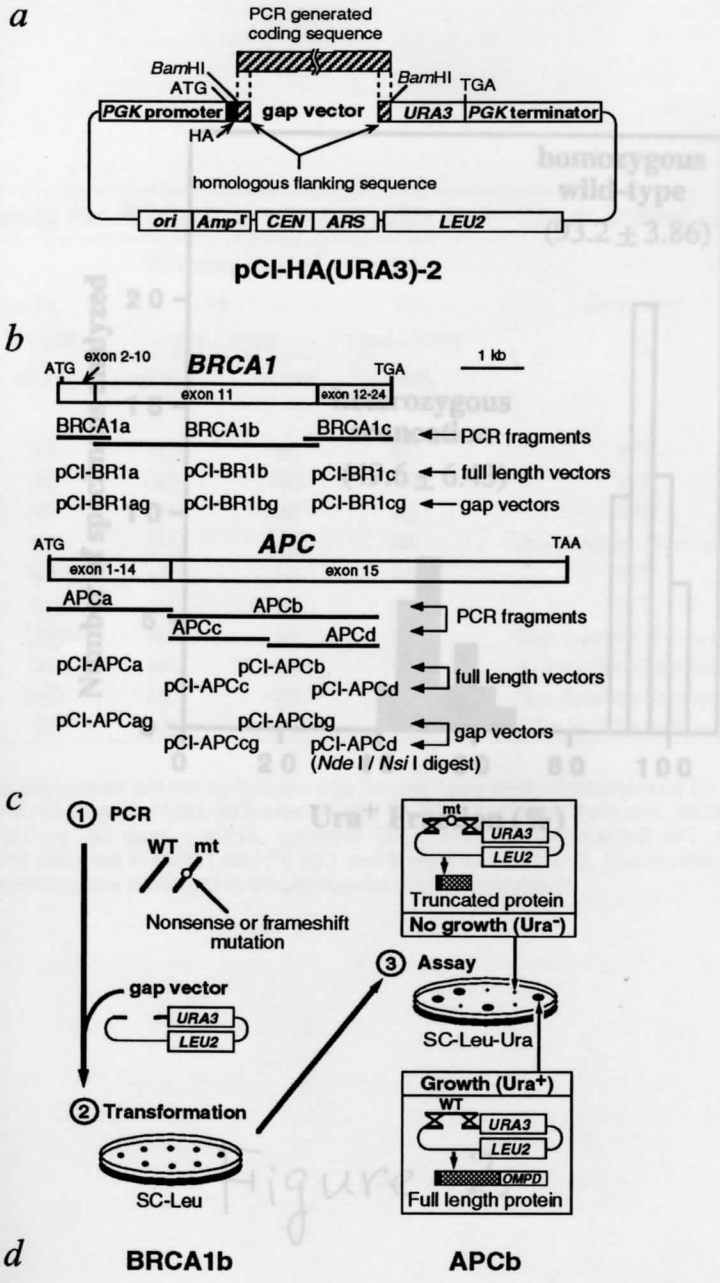
b. Sequences of *BRCA1* and *APC* chosen for analysis. cDNA or genomic fragments denoted BRCA1a-c and APCa-d were inserted in-frame into the *Bam*HI site of pCI-HA(URA3)-2, producing plasmids pCI-BR1a, b and c, and pCI-APCa, b, c and d. Plasmids, pCI-BR1ag, bg and cg, and pCI-APCag, bg and cg, are gap vectors.

c. Schematic representation of SC assay. 1. PCR amplification of cDNA or genomic fragment containing wild-type (WT) or truncated mutant (mt; nonsense or frameshift) is combined with the appropriate gap vector, which contains 100bp flanking the PCR fragment to allow for efficient homologous recombination. 2. transformation of *leu2*$^-$ and *ura3*$^-$ deficient yeast with the PCR product and gap vector yields leucine prototropic transformants that have undergone recircularization of the plasmid following homologous recombination. 3. selection of Leu$^+$ prototroph following replating in the absence of uracil distinguishes Ura$^+$ prototrophs (wild-type inserted sequence) from Ura$^-$ auxotrophs (truncation mutation). OMPD, orotidine-5'-phosphate (OMP) decarboxylase.

d. Representative SC assay for *BRCA1* (fragment BRCA1b) and *APC* (fragment APCb), showing growth of yeast transformants in the absence of uracil. In both cases, the left half-plate represents a specimen derived from a patient with a heterozygous truncating mutation, and the right half-plate is a control sample.

Fig. 2 Distribution of Ura$^+$ colonies derived from specimens with wild-type *BRCA1* and *APC* or containing a heterozygous truncating mutation.

Results of the SC assay are shown for 75 specimens for which presence (closed bar) or absence (open bar) of a truncating mutation was confirmed by nucleotide sequencing. Values in parenthesis indicate mean $\pm$ standard deviation.



Pt. 99 control

Family A control (II-2)

Figure 1

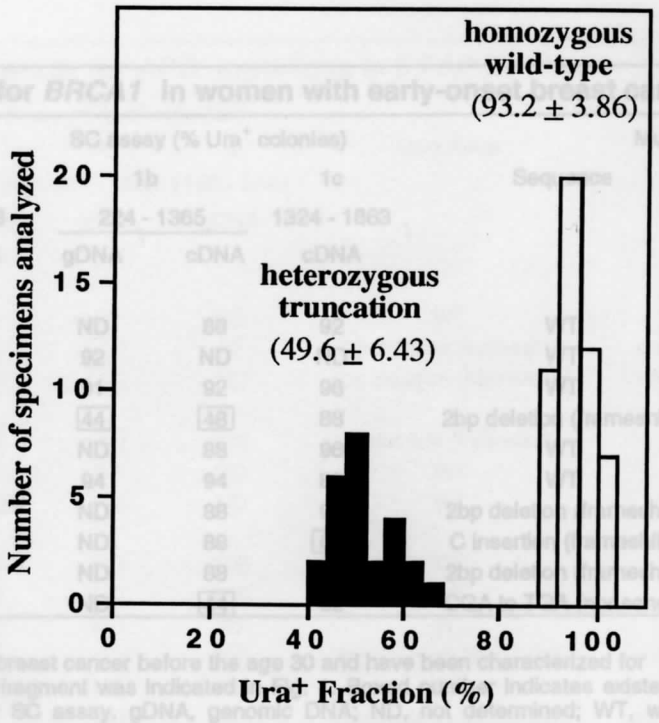

homozygous
wild-type
(93.2 ± 3.86)
heterozygous
truncation
(49.6 ± 6.43)
Number of specimens analyzed
0
5
10
15
20
0
20
40
60
80
100
Ura+ Fraction (%)

Figure 2.

Table 1 SC assay for *BRCA1* in women with early-onset breast cancer

	SC assay (% Ura$^+$ colonies)				Mutation	
BRCA1 fragment	1a	1b		1c	Sequence	Location
codons	1 - 263	224 - 1365		1324 - 1863		
PCR analysis	cDNA	gDNA	cDNA	cDNA		
Patient						
Pt. 43	92	ND	88	92	WT	
Pt. 79	ND	92	ND	ND	WT	
Pt. 84	92	91	92	96	WT	
Pt. 99	96	[44]	[48]	88	2bp deletion (frameshift)	codon 327 (exon 11)
Pt. 103	88	ND	88	96	WT	
Pt. 118	88	94	94	88	WT	
Pt. 231	[44]	ND	88	92	2bp deletion (frameshift)	codon 23 (exon 2)
Pt. 250	96	ND	88	[64]	C insertion (frameshift)	codon 1756 (exon 20)
Pt. 253	[48]	ND	88	100	2bp deletion (frameshift)	codon 23 (exon 2)
Pt. 364	96	ND	[44]	92	CGA to TGA (nonsense)	codon 563 (exon 11)

All patients are women with breast cancer before the age 30 and have been characterized for *BRCA1* mutations previously (ref.11). Location of BRCA1 fragment was indicated in Fig. 1. Boxed number indicates existence of truncating mutation in examined DNA fragment by SC assay. gDNA, genomic DNA; ND, not determined; WT, wild-type in the entire coding sequence. The mutations detected in Pt.231 and Pt. 253 are known as 185delAG. The mutation detected in Pt. 250 is known as 5382insC. These mutations are prevalent in the Ashkenazi Jewish population.

Table 2 SC assay for *APC* mutations in 6 FAP families.

		SC assay (% Ura$^+$ colonies)			Mutation	
APC fragment		b	c	d	Sequence	Location
Codons		654 - 1748	654 - 1184	1185 - 1748		
PCR analysis		gDNA	gDNA	gDNA		
Family & Indivisuals						
Family A						
	II-1	100	92	ND	WT	
	II-2	[38]	[48]	92	4bp deletion (frameshift)	codon 929 - 930 (Exon 15)
	II-3	[56]	[48]	ND	4bp deletion (frameshift)	codon 929 - 930 (Exon 15)
Family B						
	I-1	[50]	[44]	100	T insertion (frameshift)	codon 938 (Exon 15)
	II-1	96	92	ND	WT	
	II-2	88	100	ND	WT	
Family C						
	I-1	ND	ND	100	WT	
	I-2	ND	ND	100	WT	
	I-3	ND	ND	96	WT	
	I-7	[40]	92	[56]	2bp deletion (frameshift)	codon 1249 - 1250 (Exon15)
	I-8	ND	ND	92	WT	
	II-1	ND	ND	[40]	2bp deletion (frameshift)	codon 1249 - 1250 (Exon15)
	II-2	ND	ND	[48]	2bp deletion (frameshift)	codon 1249 - 1250 (Exon15)
	II-3	ND	ND	[52]	2bp deletion (frameshift)	codon 1249 - 1250 (Exon15)
Family D						
	I-1	[60]	92	[56]	5bp deletion (frameshift)	codon 1309 -1311 (Exon 15)
	II-1	ND	ND	92	WT	
	II-2	ND	ND	96	WT	
	II-3	ND	ND	88	WT	
Family E						
	I-1	[44]	96	[48]	5bp deletion (frameshift)	codon 1309 -1311 (Exon 15)
	II-1	ND	ND	92	WT	
	II-2	ND	ND	[48]	5bp deletion (frameshift)	codon 1309 -1311 (Exon 15)
	II-3	ND	ND	92	WT	
Family F						
	I-1	[57]	92	[48]	A deletion (frameshift)	codon 1322 (Exon 15)
	II-1	ND	ND	92	WT	

All individuals in the 6 FAP families are Japanese. Location of APC fragment was indicated in Fig. 1. Boxed number indicates existence of truncating mutation in examined DNA fragment by SC assay. gDNA, genomic DNA; ND, not determined; WT, wild-type in the examined coding sequence.